Hemochemistry and Hematology of the Aestivating Pond Snail *Pila globosa*

(Gastropoda : Ampullariidae)

BY

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(6 Tables)

INTRODUCTION

DEHYDRATION, QUALITATIVE and quantitative changes in energy reserves, ions, intermediary metabolites and enzyme kinetics have been reported to occur in the tissues of *Pila globosa* (Say, 1822) during aestivation (MEENAKSHI, 1956; RAGHUPATHIRAMIREDDY, 1967, and VIJAYA BRAHMANANDAM, 1972). Although the ionic composition of blood of *P. globosa* was reported by SAXENA (1957), no report is available on the hemochemistry and hematology with reference to aestivation. It is essential to examine the hemochemistry and hematology of these snails during aestivation because the blood as transporting medium plays a pivotal role in the physiology of aestivation.

MATERIAL AND METHODS

1. Aestivation of Snails

Gastropod pond snails, *Pila globosa,* were collected from the suburbs of Bangalore and stocked in laboratory aquaria. While they were kept in aquaria, they were provided with cabbage slices and *Hydrilla,* a plant on which they normally feed. After they became used to laboratory conditions, a batch of them was made to aestivate by embedding them in dry mud in large wooden boxes, 60 x 35 x 15 cm for the required period. Before burying, the snails were allowed to crawl in glass troughs overnight to dispel the mantle water. The temperature in the wooden box was maintained at 35° ± 2° C by heating with an electric lamp. Care was taken to maintain darkness by covering the bulb with tin foil. At one time about 50 to 100 snails were caused to aestivate; a new batch was started every month so that the aestivated snails were always at hand for analysis. In most cases the snails having aestivated for 6 months were selected for the investigations. Actively feeding snails from aquaria were used as controls.

2. Collection of Blood

The blood was collected from the snails by bleeding the animal at the visceral coils. Care was taken especially with the active snails to avoid mixing the mantle fluid with the blood. This was done by first draining off the mantle fluid and the animal was wiped well with filter paper before collecting the blood. The blood was used immediately for biochemical analysis.

3. Measurement of pH and osmotic pressure

The pH of the freshly collected blood was measured with a direct reading Phillips pH meter equipped with a glass and calomel electrode. The osmotic pressure of the blood was estimated by the melting point method as described by GIESE (1968).

4. Protein estimations

The total proteins in measured samples of blood was estimated by biuret method (LAYNE, 1957), after precipitating the proteins with 10% trichloroacetic acid (TCA). Albumins and globulins of the blood were separated by ammonium sulphate fractionation (COHN *et al.,* 1940) and estimated by biuret method. Bovine serum albumin (obtained ftom the Biochemical Unit, V. P. Chest Institute, New Delhi) was used as the standard for protein determinations. In some experiments, the precipitated protein after TCA treatment was dehydrated with alcohol, chloroform and ethanol (1:3) and dried with ether.

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The dried powder was weighed in a chemical balance to assess the protein content gravimetrically.

5. Non-protein fractions

The non-protein (NPN) and total a-acid contents of the TCA-treated blood filtrates were estimated by micro-kjeldhal method (Oser, 1965). The urea was estimated by nesslerization method (Oser, *op. cit.*) after hydrolysing the neutral deproteinized blood with urease.

6. Calcium-binding proteins

Per cent calcium-binding proteins were estimated in the following way according to Krishnamoorthy, 1963: 5% (wt/v) 0.25 M sucrose homogenates were prepared from the muscle of active and aestivated snails. The homogenates were centrifuged and the supernatants were dialysed overnight against 0.25 M sucrose solution. The dialysate was divided into 2 parts. In one part the protein content was estimated by the biuret method (Layne, 1957). To the other part an equal volume of 1 M $CaCl_2$ was added, thoroughly mixed and kept at 0° C for 30 minutes. The precipitate was centrifuged and suspended in distilled water, and 5% TCA was added. It was then centrifuged and the supernatant was discarded. The protein content was estimated by the biuret method. From the values obtained the percentage of proteins by calcium was calculated in each homogenate.

7. Carbohydrate composition of the blood

The deproteinized samples of the blood after TCA treatment were used to estimate the total sugars by the anthrone method (Hassid & Abraham, 1957). The blood glucose was estimated by glucostat (supplied by Worthington Biochemical Corporation) method. The blood pyruvate was estimated by the 2, 4 - dinitrophenyl hydrazine method (Oser, 1965) and the lactate by colorimetric-enzymatic method (Bruns & Bergmeyer, 1965).

8. Ionic composition of the blood

The chloride content of deproteinized blood was estimated titrimetrically (Milton & Waters, 1949). Blood calcium content was estimated by the method of Clark - Collip modifications of Kramer - Tisdall method (Oser, 1965). Magnesium was estimated by microtitration after its separation as the salt of 8-hydroxyquinoline (Milton & Waters, 1949). The blood sodium was determined by the method of Weinback (Oser, *op. cit.*). Potassium was determined by the method of Looney and Dyer (Oser, *op. cit.*).

9. Blood count

The blood cell count was pursued with the help of a hemocytometer using snail Ringer (Coles, 1969) as the dilution medium.

10. Assay of blood enzymes

The LDH activity of the blood was estimated spectrophotometrically (Neilands, 1957) by following the NA DH formation when the sample is incubated with sodium lactate and NAD. The glutamic-pyruvate transaminase (GPT) activity of the blood was estimated by the method of Reitman & Fraenkel (1957). The glycerol dehydrogenase activity of the blood was assayed spectrophotometrically according to Neilands (*op. cit.*), using glycerol and NAD in the assay medium. The glutamic dehydrogenase was assayed in the same way with glutamate and NAD in the assay medium.

RESULTS

As is evident from Table 1, aestivation in *Pila globosa* does not affect the pH of the blood, but the osmotic pressure was elevated 3 to 4 fold. Elevation in osmotic pressure may be due to an increase in soluble proteins, sugars, amino-acids or the salt composition of the blood, as these substances contribute to the osmotic relations of blood.

Table 1

Changes in the pH and Osmotic Pressure of Blood of Aestivating *Pila globosa*

	pH	Osmotic pressure atmospheres
Active snails	7.90±0.18 (12)	5.4 ±0.6 (6)
Aestivated snails	7.97±0.16 (12)	19.82±1.8 (6)
Incidence of change on aestivation	no change t=0.9660 p>0.1	Increase t=33.49 p<0.001

values are mean ± S. D.; number in parentheses is number of observations

Table 2 presents the data on the changes of nitrogenous compounds in the blood of *Pila globosa* during aestivation. Total blood proteins did not vary, whereas the total non-protein nitrogen, urea and total amino-acids, increased on

Table 2

Quantitative Changes in the Total Protein, Non-Protein Nitrogen Content and different Fractions of Proteins of *Pila globosa* during Aestivation

No.	Constituent	Active	Aestivated	Incidence of change on aestivation
1	Total protein gravimetric method (mg/ml) (6)	42 ± 1	39 ± 0.5	t=6.003 decrease $p < 0.001$
	Folin's method (mg/ml) (6)	40 ± 4.79	36 ± 3.05	t=1.579 no change $p > 0.1$
2	Total amino acid content glycine equivalents μg/ml) (6)	673 ±102	1246 ±249	t=4.246 increase $p < 0.01$
3	Total non-protein nitrogen (mg%) (6)	5.8± 0.66	9.6± 0.42	t=10.72 increase $p < 0.001$
4	Total albumin (mg/ml) (6)	21 ± 2.1	30 ± 1.9	t=7.109 increase $p < 0.001$
5	Total globulins (mg/ml) (6)	19 ± 1.5	7.8± 1.1	t=15.92 decrease $p < 0.001$
6	Urea (μg/ml) (6)	168 ± 33	216 ± 24	t=2.595 increase $p < 0.01$
7	Dry matter (μg/ml) (6)	42 ± 3.7	41 ± 1.8	t=0.8983 no change $p > 0.1$

values are mean ± S. D.; number in parentheses is number of observations

Table 3

Changes in the Ionic Composition of the Blood of *Pila globosa* during Aestivation

Ion	Active	Aestivated	Incidence of change on aestivation
Calcium (mg/100 ml)	22 ± 5.29 (7)	56± 12.9 (8)	t=6.023 increase $p<0.001$
Magnesium (mg/100 ml)	15 ± 2.75 (7)	28± 10.08 (7)	t=3.114 increase $p<0.01$
Sodium (mg/100 ml)	1890 ±175 (6)	3038± 233 (6)	t=8.825 increase $p<0.001$
Potassium (mg/100 ml)	48 ± 10.4 (6)	62± 13.9 (6)	t=1.806 no change $p>0.1$
Chloride (mgCl/100 ml)	119.5± 4.8 (6)	121± 7.9 (6)	t=0.3626 no change $p>0.1$

values are mean ± S. D.; number in parentheses is number of observations

Table 4

Per cent Calcium-binding Proteins of Blood and Sucrose Soluble Fractions of Tissues of *Pila globosa* during Aestivation

Protein	Active snail	Aestivated snail	Incidence of change on aestivation
Blood proteins	5.23±0.93 (6)	5.98± 0.67 (6)	no change
Foot muscle proteins	17.58±6.84 (8)	44.18±11.71 (8)	increase
Mantle muscle proteins	19.32±5.68 (5)	48.38± 5.69 (5)	increase

values are mean ± S. D.; number in parentheses is number of observations

aestivation. Total albumins of the blood increased in contrast to total globulins. The dry matter did not change per unit volume of blood during aestivation.

Table 3 presents the changes in ionic composition of blood due to aestivation. Calcium, magnesium. and sodium concentrations increased, whereas chloride and potassium did not show any change on aestivation. The calcium-binding proteins of the foot and mantle increased but the blood Ca^{++}-binding proteins did not vary in concentration (Table 4).

Table 5 presents the quantitative changes in the carbohydrate composition of the blood due to aestivation. Total

Table 5

Quantitative Changes in the Carbohydrate Composition of Blood of *Pila globosa* during Aestivation

Constituent	mg/ml or μM/ml		Incidence of change on aestivation
	Active	Aestivated	
1 Total sugars	1.97± 0.21 (8)	1.57± 0.26 (8)	t=3.432 p<0.01 decrease
2 Glucose	1.58± 0.39 (7)	1.16± 0.026(6)	t=2.437 p<0.01 decrease
3 l (+) lactate (μM/ml)	57 ±19.6 (4)	44 ±18.56 (4)	t=0.834 p>0.1 no change
4 Pyruvate (μg/ml)	20 ± 6.6 (8)	7.5 ± 1.4 (7)	t=4.576 p<0.01 decrease

values are mean ± S. D.; number in parentheses is number of observations

sugars, glucose and pyruvate decreased on aestivation, whereas the lactate did not vary much, contrary to Meenakshi's (1956) findings.

Table 6

Specific Activities of Certain Enzymes in the Blood of *Pila globosa* during Aestivation

Specific Activity	Active	Aestivated
1 LDH (units)	980±21 (6)	568 ±32 (6)
2 GPT (μg pyruvate/mg protein/hr)	12± 2.6 (6)	5.6± 1.4 (6)
3 Glycerol dehydrogenase (units)	244±42 (6)	686 ±48 (6)
4 Glutamic dehydrogenase (units)	128±34 (6)	462 ±18 (6)

values are mean ± S. D.; number in parentheses is number of observations

Table 6 illustrates that the specific activities of blood LDH and GPT decreased on aestivation in contrast to glycerol dehydrogenase and glutamic dehydrogenase.

Hematologically, blood shows variations in composition and also in differential count. Two types of cells, large and small, were identified. The large cell count was more or less the same (*i. e.*, 2.1 ± 0.09 thousands per ml) in aestivated snails, but the small cell count decreased and was 23.6 ± 2.16 thousands per ml in aestivated snails.

DISCUSSION

Dehydration has been noted by Raghupathiramireddy (1967) and Vijaya Brahmanandam (1972) in the liver, mantle, and foot muscle of *Pila globosa* during aestivation. The present results indicate that there is no dehydration in the blood since the total proteins and total dry matter did not vary on aestivation (Table 2). Albumins, being water soluble proteins, are known to imbibe more water than the globulins (Mahler & Cordes, 1966).

Increase in albumins (Table 2) on aestivation may help to prevent desiccation by way of water retention through imbibition.

During aestivation accumulation of nitrogenous wastes in the tissues occur (Rhagupathiramireddy & Swami, 1963). Concomitantly, accumulation of NPN reserves and urea content were observed in the present results (Table 2). The accumulation of these substances may be due to the lack of a medium for excretion. Increase in total blood amino acids on aestivation (Table 2) may suggest either higher degradation of tissue proteins or blood globulins (Table 2) and disintegration of smaller red cells.

Increase in ionic composition, like Ca^{++}, Mg^{++}, and Na^{++} on aestivation (Table 3) has its significance in contributing to the elevation of blood osmotic pressure (Table 1) and also in obviating the osmotic effects at tissue level which may prevail due to dehydration. Increase in Mg^{++} in body fluids of aestivating snails (Meenakshi, 1956; Raghupathiramireddy, 1967) is known. These authors suggested that the dormancy of the snail may be due to the

anaesthetic property of the Mg^{++}. Increase in Ca^{++} may have significance in maintenance of calcium-binding proteins in tissues which are increased in concentration during aestivation (Table 4). It is probable that Ca^{++} may be secreted from the shell or mantle into the circulating fluids during dormancy which may cause this increase. Increase in ions did not affect the pH of the blood (Table 1).

The results on carbohydrate composition of the blood of aestivating animals indicate the metabolic patterns they experience. Decrease in total sugars, glucose and pyruvate levels (Table 5) may be due to the effect of starvation during aestivation. Decreased LDH activity (Table 6) reduced the oxidation of lactate and the pyruvate undergoes transaminations (Table 6) and hence the pyruvate level is decreased during aestivation. There is evidence (Krishnamoorthy & Vijaya Brahmanandam, 1970) that the reserve fats of tissues offer a source of energy.

SUMMARY

1. Hemochemistry and hematology of the aestivating pond snail *Pila globosa* with reference to aestivation was studied.
2. The pH of the blood was not affected as a consequence of aestivation, but the osmotic pressure was elevated 3 to 4-fold.
3. There was no dehydration in the blood as the total proteins and total dry matter did not vary on aestivation.
4. The carbohydrate composition of the blood of aestivating snails indicated the metabolic patterns they experience.
5. Ca^{++}, Mg^{++} and Na^{+} concentrations increased, whereas chloride and potassium did not show any change on aestivation.

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Literature Cited

Brahmanandam, Vijaya
1972. Some biochemical studies on gastropod muscle proteins with particular reference to aestivation. Ph. D. dissertat. submitted to Bangalore Univ., India
Bruns, F. H. & H. V. Bergmeyer
1965. Methods of enzymatic analysis. Acad. Press, New York
Cohn, E., T. L. McMeekin, J. L. Oncley, J. M. Newell & W. L. Hughes
1940. Preparation and properties of serum and plasma proteins. I. Size and charge of proteins separating upon equilibration across membranes with ammonium sulphate solutions of controlled pH, ionic strength and temperature. Journ. Amer. Chem. Soc. **62:** 3386 - 3393
Coles, G. C.
1969. The termination of aestivation in the large freshwater snail *Pila ovata* (Ampulariidae) - II. *in vitro* studies. Compar. Biochem. Physiol. **29:** 373 - 379
Giese, A. C.
1968. Temperature as a factor in the cell environment. *in*: Cell physiology, 3rd ed., W. B. Saunders Co., Philadelphia, pp. 235 - 251
Hassid, W. Z. & S. Abraham
1957. Chemical procedures for analysis of polysaccharides. *in*: Methods in Enzymology III: 35 - 50. S. P. Colowick & N. O. Kaplan, eds. Acad. Press, New York
Krishnamoorthy, R. V.
1963. Charge properties of cell contents in relation to hydrogen ion concentration. Ph. D. dissert. subm. to Sri Venkateswara Univ., Tirupati, A. P, India
Krishnamoorthy, R. V. & Vijaya Brahmanandam
1970. Hepatopancreatic lipolytic activity in the aestivated pond snail *Pila globosa*. Indian Journ. Exper. Biol. **8:** 145 - 146
Layne, E.
1957. Spectrophotometric and turbidimetric method for measuring proteins. *in*: Methods in Enzymology III: 447 - 454, S. P. Colowick & N. O. Kaplan, eds. Acad. Press, New York
Mahler, H. R. & E. H. Cordes
1966. Biological chemistry. Harper & Row, New York, 872 pp.
Meenakshi, V. R.
1956. The physiology of hibernation of the apple snail *Pila virens* (Lamarck). Curr. Sci. (India) **25** (10): 321 - 322
Milton, R. F. & W. A. Walters
1949. Methods of quantitative micro-analysis. Edward Arnold & Co. London
Neilands, J. B.
1957. Lactic dehydrogenase of heart muscle. *in*: Methods in Enzymology II: 449 - 454. S. P. Colowick & N. O. Kaplan, eds. Acad. Press, New York
Oser, B. L., ed.
1965. Hawk's physiological chemistry, 13th ed., McGraw-Hill Book Co., New York, 1472 pp.
Raghupathiramireddy, S.
1967. Respiratory enzymes during aestivation of the Indian apple snail *Pila globosa*. Life Sciences **6:** 341 - 345
Raghupathiramireddy, S. & K. S. Swami
1963. Distribution of uric acid in the soft parts of the amphibious snail *Pila globosa*. Canad. Journ. Biochem. **45:** 600 - 607
Reitman, S. & S. Frankel
1957. A colorimetric method for the determination of glutamic oxaloacetate and glutamic pyruvate dehydrogenase. Amer. Journ. Clin. Pathol.: 28 - 56
Saxena, B. B.
1957. Inorganic ions in blood of *Pila globosa* (Swainson). Physiol. Zool. **30:** 161 - 164